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Thermal acclimation and stress in the American lobster, *Homarus americanus*: equivalent temperature shifts elicit unique gene expression patterns for molecular chaperones and polyubiquitin

Jeffrey L. Spees,* Sharon A. Chang, Mark J. Snyder, and Ernest S. Chang

Bodega Marine Laboratory, University of California, PO Box 247, Bodega Bay, CA 94923, USA

Abstract Using homologous molecular probes, we examined the influence of equivalent temperature shifts on the in vivo expression of genes coding for a constitutive heat shock protein (Hsc70), heat shock proteins (Hsps) (Hsp70 and Hsp90), and polyubiquitin, after acclimation in the American lobster, *Homarus americanus*. We acclimated sibling, intermolt, juvenile male lobsters to thermal regimes experienced during overwintering conditions (0.4 ± 0.3°C), and to ambient Pacific Ocean temperatures (13.6 ± 1.2°C), for 4–5 weeks. Both groups were subjected to an acute thermal stress of 13.0°C, a temperature shift previously found to elicit a robust heat shock response in ambient-acclimated lobsters. Animals were examined after several durations of acute heat shock (0.25–2 hours) and after several recovery periods (2–48 hours) at the previous acclimation temperature, following a 2-hour heat shock. Significant inductions in Hsp70, Hsp90, and polyubiquitin messenger RNA (mRNA) levels were found for the ambient-acclimated group. Alternatively, for the cold-acclimated group, an acute thermal stress over an equivalent interval resulted in no induction in mRNA levels for any of the genes examined. For the ambient-acclimated group, measurements of polyubiquitin mRNA levels showed that hepatopancreas, a digestive tissue, incurred greater irreversible protein damage relative to the abdominal muscle, a tissue possessing superior stability over the thermal intervals tested.

INTRODUCTION

Many ectothermic organisms acclimate and survive over wide temperature ranges. Lacking the heat-producing capacity of endotherms, their metabolism is at the mercy of temperature changes dictated by seasons and disturbances. These thermal fluctuations are not trivial. Critical biological processes, including development, growth, and fitness, are all temperature dependent. Responding to thermal variation in the environment, numerous organisms have evolved biochemical adaptations to buffer against temperature changes. These adaptations include homeoviscous and homeophasic alteration of cell membranes (Vigh et al 1998), enhanced protein stability because of specialized amino acid sequence (eg, *Thermus aquaticus* DNA polymerase) (Somero 1995), elevated concentrations of intracellular osmolytes, such as glycerol, sorbitol, trehalose, and glucose (Yancey et al 1982), antifreeze glycoproteins (Cheng and Chen 1999), and inducible stress proteins (molecular chaperones, heat shock proteins [Hsps]) (Feder and Hofmann 1999).

Production of Hsps is a common cellular response to thermal stress. In addition to chaperoning nascent polypeptides into their native structures, Hsps are known to protect against thermal stress by refolding denatured proteins and preventing protein aggregation (see reviews in Morimoto et al 1990; Hartl 1996; Nover and Scharf 1997; Bukau and Horwich 1998). Some Hsps such as

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*Current address: Center for Gene Therapy, Tulane University Health Sciences Center, New Orleans, LA 70112, USA.

Correspondence to: Ernest S. Chang, Tel: 707 875-2061; Fax: 707 875-2009; E-mail: eschang@ucdavis.edu.

Hsp104 in yeast, *Saccharomyces cerevisiae* (Sanchez and Lindquist 1990), are required for induced thermotolerance, a process by which an organism previously exposed to a sublethal temperature can subsequently survive a normally lethal heat stress (Schlesinger et al 1982; Lindquist and Craig 1988; Clegg et al 1998).

Intense or prolonged thermal stress can permanently damage proteins, promoting ubiquitination and degradation via the 26S proteasome (Varshavsky 1997; Mykles 1998).

Whereas Hsp expression may be viewed as a measure of reversible protein damage, ubiquitin production and conjugation are considered signs of irreversible protein damage (Parsell and Lindquist 1993; Hofmann and Somero 1996). Through a series of enzymatic reactions, the 76 amino acid ubiquitin polypeptide is covalently bound to damaged proteins or regulatory proteins, such as transcription factors, cell cycle regulators, kinases, phosphatases, and tumor suppressors (Bonifacino and Weissman 1998). The presence and patterning of ubiquitin moieties determine the specificity of the 26S proteasome, which is signaled to degrade particular peptide subunits or entire proteins (Hochstrasser 1996; Varshavsky 1997).

In the American lobster, *Homarus americanus*, multiple ubiquitin coding regions are transcribed as a single transcript (polyubiquitin), which is posttranslationally processed into individual ubiquitin moieties (Shean and Mykles 1995). Here, we use polyubiquitin gene expression as an indirect measure of protein degradation. As the number of proteins permanently damaged by a particular environmental insult increases, greater numbers of polyubiquitin transcripts are required for ubiquitin production to support the degradative pathway. Polyubiquitin expression is correlated with the degradation of myofibrillar proteins during premolt claw atrophy in the land crab, *Gecarcinus lateralis* (Shean and Mykles 1995), and in *H. americanus* (Spees, personal communication).

Because of their remarkable ability to maintain homeostasis and metabolism during long-term thermal acclimatization or acclimation, lobsters are unique organisms for studying thermal acclimation and stress. Lobsters can acclimate and survive at temperatures ranging from −1.0°C to 30.5°C (Lawton and Lavalli 1995). Although one might expect the lobster to become dormant at temperatures below 5°C (at these temperatures growth is minimal), lobsters are known to be active and even to mate at temperatures in the 0–2°C range. In fact, individuals that reach premolt (stage D_1) before declining autumn temperatures may continue the molting process, eventually shedding the exoskeleton at temperatures as low as 0°C (Waddy et al 1995).

Throughout the long-term acclimatization to winter thermal regimes, lobsters are likely to change metabolically, down-regulating DNA replication, and altering protein synthesis and enzyme reaction rates. Modifications in both subunit expression and isozyme patterns have been studied in response to temperature changes (Hochachka and Somero 1984; Somero 1995). To maintain both the function and efficiency of enzymatic reactions during thermal acclimation, the structures of some requisite proteins such as carp (*Cyprinus carpio*) myosin ATPase are known to display specific temperature-dependent differences. Myosins from 30°C-acclimated carp are about 4 times as thermostable as those from 10°C-acclimated carp (Hwang et al 1990). Similar types of conformational changes could occur during the long-term thermal acclimatization of overwintering lobsters. Trausch (1976) found that temperature-mediated changes in the configuration of lactate dehydrogenase profoundly influenced its kinetics in the abdominal muscle (AM) of the lobster *Homarus gammarus*. Proteins that undergo flexibility changes, conformational adjustments, or isozyme exchanges over different thermal regimes are likely to differ in stability or lability (or both) characteristics (Johnston et al 1973; Hashimoto et al 1982; Hwang et al 1990). With regard to these observations and those concerning plasticity in the heat shock response for marine ectotherms (Dietz and Somero 1992; Dietz 1994; Roberts et al 1997; Tomanek and Somero 1999), we examined thermal acclimation and stress in *H. americanus*, a eurythermal organism adapted to survive cold overwintering temperatures, as well as thermal shifts during spring and summer reproductive migrations (Crossin et al 1998). Following acclimation of lobsters to ambient and cold temperatures, we examined whether acute temperature shifts over equivalent intervals (13.0°C) would elicit analogous signs of protein denaturation such as Hsp and polyubiquitin expression.

MATERIALS AND METHODS

Animal care

Juvenile lobsters (sibling, intermolt males; mean weight 85.3 ± 17.2 g) reared at the Bodega Marine Laboratory (BML) were acclimated (4–5 weeks) to cold (0.4 ± 0.3°C, $n = 44$) and ambient (13.6 ± 1.2°C, $n = 44$) temperatures. Ambient-acclimated lobsters were maintained in a flow-through aquaculture system, held in individual compartments, and fed shrimp 3 times weekly. Detailed descriptions of the aquaculture system and lobster culture techniques at BML are reviewed elsewhere (Chang and Conklin 1993; Conklin and Chang 1993).

Cold-acclimated lobsters were held in 2-L glass jars filled with seawater in a temperature-controlled cold room. Each jar was supplied with an airstone, and water was changed once weekly. Water was changed twice during the first week of acclimation to prevent possible

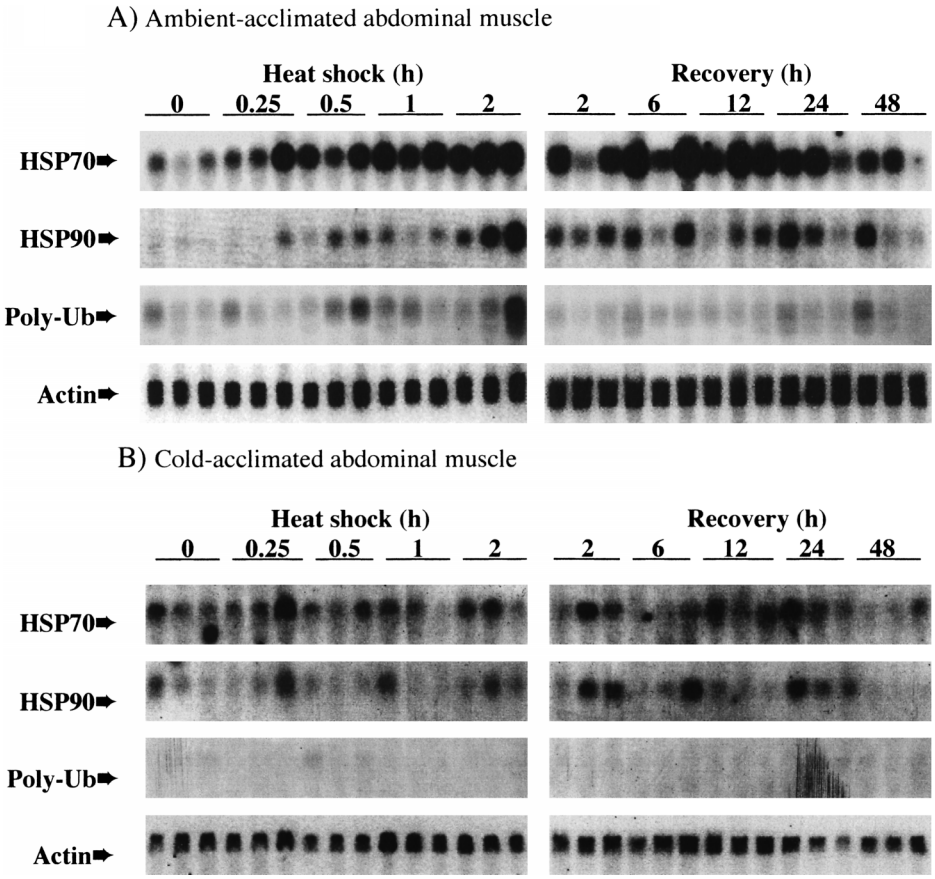

Fig 1. Abdominal muscle messenger RNA levels for Hsp70, Hsp90, polyubiquitin (Poly-Ub), and actin (indicator of equal loading) in (A) ambient-acclimated and (B) cold-acclimated lobsters during heat shock and recovery. Data for each acclimation group represent a single gel that was transferred and probed with several complementary DNAs. Polyubiquitin transcripts were hardly detectable in the cold-acclimated group; we did not attempt to quantify these latter data.

buildup of nitrogenous wastes. These animals were fed peeled shrimp, ad libitum, once weekly (before the water change). Generally, animals would not eat more than once a week, likely because of decreased metabolic requirements. Initial transfer to the cold room occurred in the 2-L glass jars filled with ambient-temperature seawater. Over the next 12 hours, the water temperature gradually equilibrated to ~0.4°C. Temperatures were monitored and recorded daily for both the ambient- and cold-acclimated groups.

Thermal stress

Both groups received an acute thermal stress (an elevation of 13.0°C) for 2 hours. Ambient-acclimated lobsters were heat shocked in aerated seawater in 2-L glass jars, previously equilibrated in a temperature-controlled (±0.1°C) water bath. Cold-acclimated lobsters were heat shocked by returning them directly to compartments in the flow-through seawater system (13.4°C).

Four animals from each group were sacrificed immediately at several time points during the heat stress (0.25 hour, 0.5 hour, 1 hour, and 2 hours) and after several recovery periods at the previous acclimation temperature, after the 2 hours of heat shock (2 hours, 6 hours, 12 hours, 24 hours, 48 hours, and 96 hours recovery). Four unstressed control animals were also sacrificed from each acclimation group. For all time points and controls, several samples of AM and hepatopancreas (HP) (=midgut gland) were dissected, frozen in liquid N_2, and stored at −70°C.

Control experiments

We ran two separate control experiments to look for potential jar effects for the animals that were acclimated in the cold room. To determine whether long-term cold room acclimation in the 2-L jars would affect basal mRNA levels for the stress-responsive genes we examined, 8 lobsters were maintained in the cold room, as described earlier, for 4 weeks. In the place of a flow-through system in the cold room (which was not available), we housed 4 of the animals in 40-L aquarium tanks that were fitted with airstones (these tanks have approx-

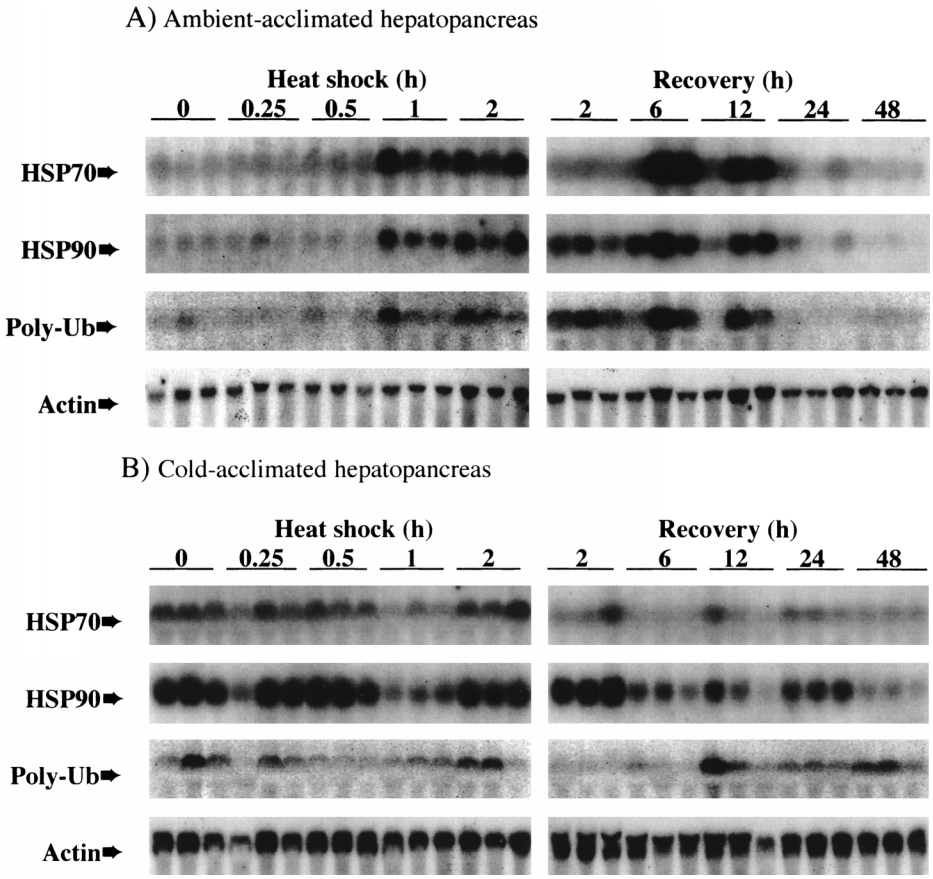

Fig 2. Hepatopancreas messenger RNA levels for Hsp70, Hsp90, polyubiquitin (Poly-Ub), and actin (indicator of equal loading) in (A) ambient-acclimated and (B) cold-acclimated lobsters during heat shock and recovery. Data for each acclimation group represent a single gel that was transferred and probed with several complementary DNAs.

imately 20× the volume of the 2-L jars). Water changes (3/4) were performed every other day for the aquarium tanks to simulate a flow-through environment. Four different animals were maintained in 2-L jars, with water changes performed once weekly, as described earlier. After 4 weeks of acclimation, and at the end of a weekly cycle (ie, no water change for the animals in 2-L jars), all 8 lobsters were sacrificed, and AM and HP were dissected, frozen in liquid N_2, and stored at −70°C. After 4 weeks of acclimation, the seawater pH from either the 2-L jars or the aquarium tanks was similar to the seawater pH of BML's flow-through system (~8.0).

We also examined whether heat shocking the cold-acclimated lobsters by returning them to the flow-through system would be different from heat shocking them in the 2-L jars. Eight lobsters were acclimated for 4 weeks in the 2-L jars, as described earlier. Four of these animals were removed from the cold room and heat shocked by direct placement in the flow-through system in individual compartments for 2 hours. Four 2-L jars (aerated and containing fresh seawater) were submerged in the flow-through system up to an inch below the jar rim and equilibrated to the temperature of the system. Four animals were then transferred from the cold room to the 4 jars and heat shocked for 2 hours. The temperature of the flow-through system was 13.6°C. All 8 lobsters were sacrificed, and AM and HP were dissected, frozen in liquid N_2, and stored at −70°C.

Molecular probe isolation and Northern analysis

Total RNA was isolated from juvenile *H. americanus* HP (Totally RNA kit, Ambion, Austin, TX, USA), and 5 μg was reverse transcribed at 37°C using an oligo-dT_{15} primer (Superscript reverse transcriptase, Stratagene protocol). This complementary DNA (cDNA) was used as template for polymerase chain reaction (PCR). A 500-bp partial clone for lobster *Hsp70* was amplified using the following degenerate primers (Cochrane et al 1994): 5′ GC[GCT] AAGAA[TC]CA[AG]G[TC][TC]G[ACGT]ATGAAC 3′ and 5′ GT[AGT]G[AC][CT]TT[AGC]AC[AGC]TC[AG]AAGAT 3′. Using the same template, a 380-bp partial clone for lobster *Hsp90* was also isolated using a 5′ GGGAAGCACGA-

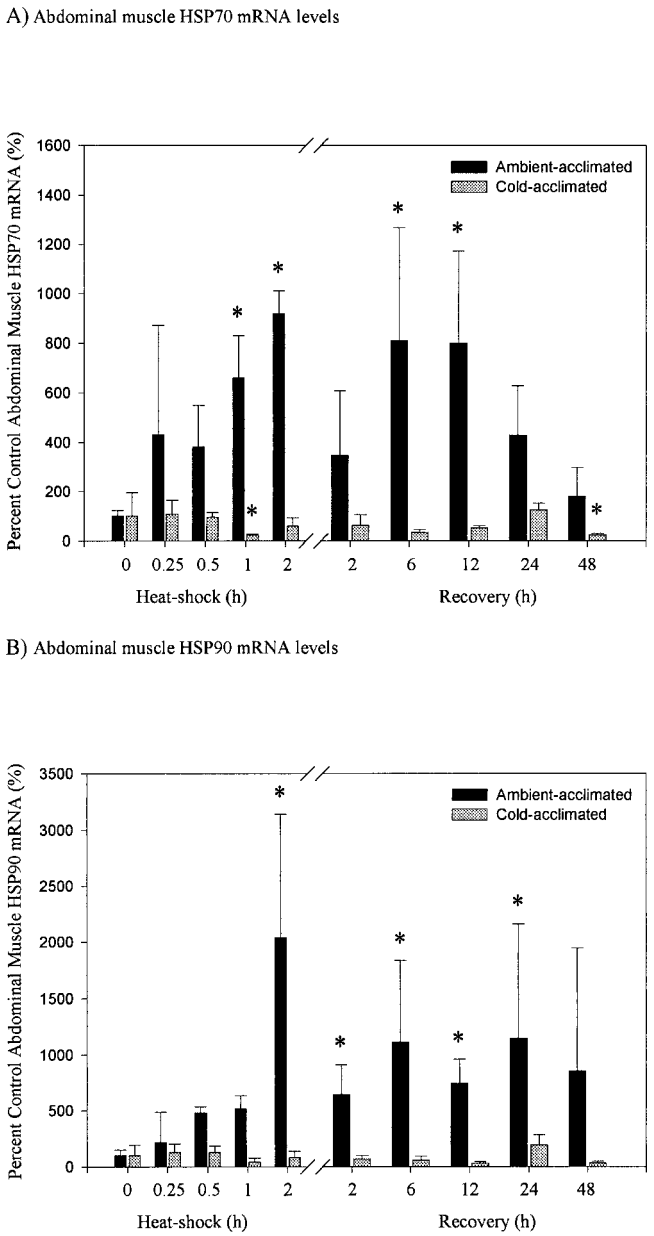

Fig 3. Comparisons of (A) Hsp70 and (B) Hsp90 messenger RNA (mRNA) levels in abdominal muscle of ambient- and cold-acclimated lobsters during heat shock and recovery. Data are normalized against actin levels (to control for equal loading) and presented as percent control expression levels. $n = 3$ for all time points. Error bars represent 1 SD of the mean. Asterisks denote statistical significance from control (no heat shock) mRNA levels within an acclimation group (* $P < 0.05$).

GGAGCGGAGG 3′ forward primer and an oligo-dT_{15} reverse primer.

Strong nucleotide sequence similarity between Hsp70 and the constitutive Hsp Hsc70 allowed the degenerate Hsp70 primers, described earlier, to anneal and amplify large segments of an Hsc70 cDNA in an anchored PCR approach. This involved the dilution, boiling, and centrifugation of an aliquot of a lobster HP cDNA library. One microliter of this preparation served as a template for separate 50 μL PCR reactions, utilizing the Hsp70 forward and reverse primers, and SP6 and T7 RNA vector promoter primers. A 2100-bp fragment of *Hsc70,* derived from the Hsp70 forward and SP6 reverse reaction, was cloned and subsequently digested with *Eco*RI to yield a 600-bp fragment. This fragment was then subcloned into the pCR2 vector (Invitrogen, Carlsbad, CA, USA). Because it consists largely of sequence from the 3′-untranslated region of lobster Hsc70, this 600-bp gene segment was used to measure Hsc70 expression specifically as this probe does not hybridize to the Hsp70 sequence (data not shown).

All PCR reactions were performed under the following conditions: 95°C initial denature, 3 minutes (1 cycle); 95°C, 1 minute; 56°C, 1.5 minutes; 72°C, 2 minutes (35 cycles); 72°C final extension, 10 minutes (1 cycle), using *Taq* DNA polymerase (PCR buffer, 2.5 mM Mg^{2+}, GIBCO, Grand Island, NY, USA). PCR products were gel-purified (Qiagen kit, Valencia, CA, USA) and cloned into the pCR 2.1 or pCR2 vectors (Invitrogen). PCR products were sequenced by the DNA sequencing facility, University of California, Davis, CA, USA or by Davis Sequencing, Davis, CA, USA. NETBLAST searching (NCBI) was used to confirm the identities of PCR products.

Total RNA was isolated from the dissected AM and HP samples (RNAgents kit, Promega, Madison, WI, USA), quantified with a spectrophotometer, and equally loaded (15 μg AM total RNA, 25 μg HP total RNA) onto denaturing 1% agarose gels. These gels were washed (15 minutes, H_2O with 0.1% diethyl pyrocarbonate (DEPC), St Louis, MO, USA) and blotted overnight onto nylon membranes (Magnagraph, MSI). Following UV cross-linking (UV Stratalinker 1800), blots were prehybridized (2 hours) in 5× SSPE (75 mm NaCl, 50 mm NaH_2 PO_4, 5 mm ethylene-diaminetetraacetic acid, 0.1% DEPC) buffer, 50% (w/v) formamide, 5× Denhardt's, 1% sodium dodecyl sulfate (SDS), and 100 μg/mL sheared salmon sperm DNA. The partial lobster *Hsp70* clone was ^{32}P-labeled (Prime-It RmT, Stratagene, La Jolla, CA, USA), added directly to the prehybridization solution, and allowed to hybridize overnight at 42°C. Following hybridization, the blots were washed twice with 2× SSPE and placed on a film for 2 days at −70°C.

Following exposure of the film, the blots were stripped with several washes (0.1× standard saline citrate, 0.1% SDS, 65°C) until the background was minimal and prehybridized, hybridized, and washed as described earlier, except that a partial lobster *Hsp90* or polyubiquitin (600 bp; gift of Prof D.L. Mykles) cDNA probe was added. To check for equal loading of RNA, the blots were probed with a 700-bp partial cDNA probe for lobster (*H. gammarus*) actin (Harrison and El Haj 1994). Separate blots were run to examine Hsc70 mRNA levels with an iden-

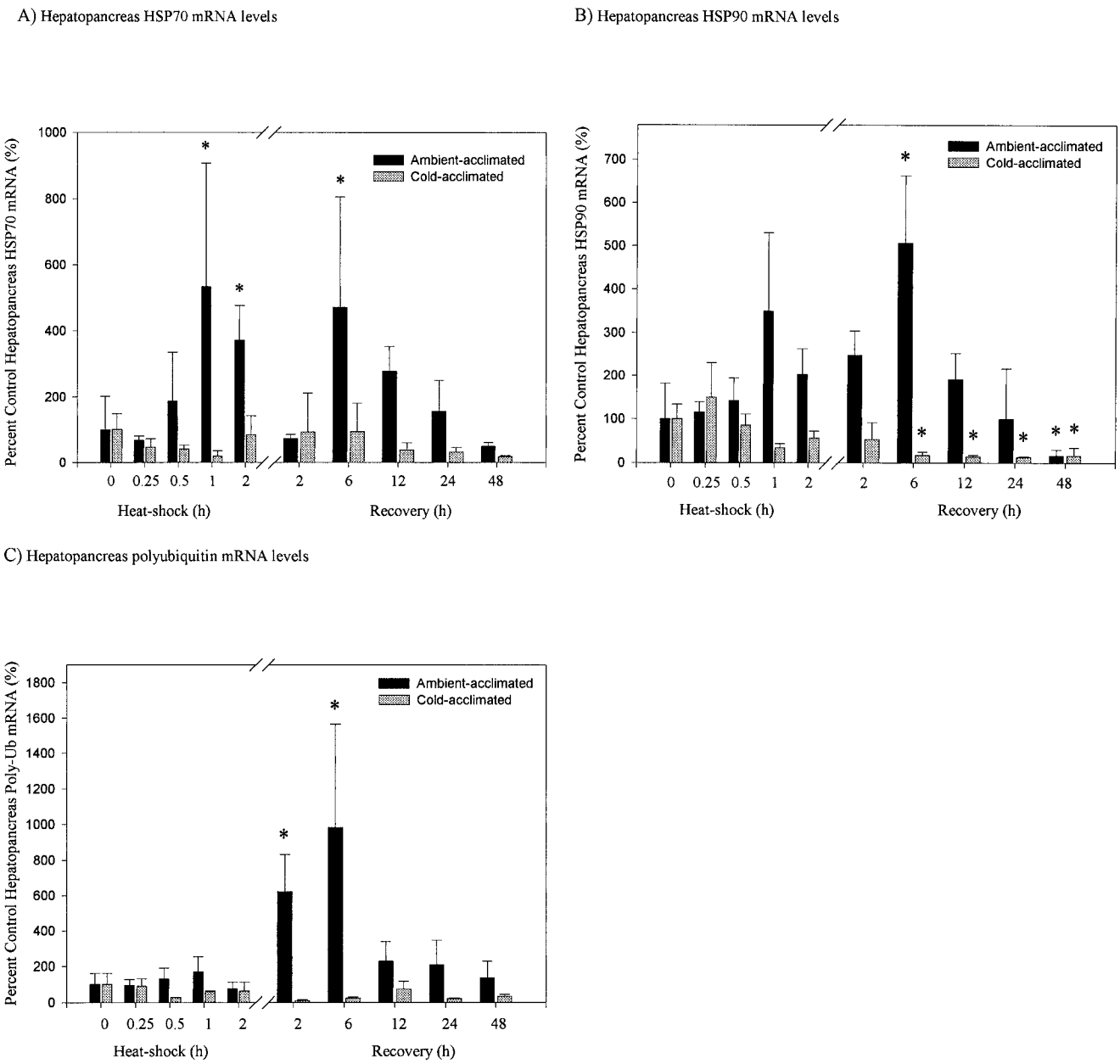

Fig 4. Comparisons of (A) Hsp70, (B) Hsp90, and (C) polyubiquitin messenger RNA (mRNA) levels in hepatopancreas of ambient- and cold-acclimated lobsters during heat shock and recovery. Data are normalized against actin levels (to control for equal loading) and presented as percent control expression levels. $n = 3$ for all time points. Error bars represent 1 SD of the mean. Asterisks denote statistical significance from control (no heat shock) mRNA levels within an acclimation group (* $P < 0.05$).

tical protocol, as described earlier, using the 600-bp partial clone specific for *Hsc70* as a probe. Films were scanned on a high-resolution scanner, and densitometry was performed with Scion Image 2.1 software.

RESULTS

Hsp70 gene expression

There were marked differences in Hsp70 gene expression between ambient- and cold-acclimated animals. These differences were apparent in both AM (Fig 1) and HP (Fig 2). All gene expression data were $\log_{10}$ transformed prior to one-way analysis of variance (ANOVA) to normalize variance. Dunnett's test was used to compare treatment values with control values following significant ANOVA results.

In ambient-acclimated AM, significant differences in Hsp70 mRNA levels were detected (ANOVA; $P = 0.011$; Fig 3A). Further testing of heat shock and recovery values vs control values indicated significant increases in Hsp70 mRNA levels following 1 hour and 2 hours of heat shock,

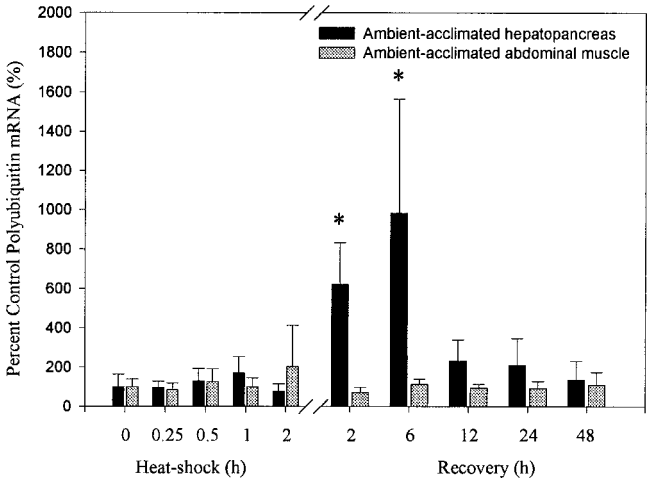

Fig 5. Comparison of polyubiquitin messenger RNA (mRNA) levels in hepatopancreas and abdominal muscle of ambient-acclimated lobsters during heat shock and recovery. Data are normalized against actin (to control for equal loading) and presented as percent control expression levels. $n = 3$ for all time points. Error bars represent 1 SD of the mean. Asterisks denote statistical significance from control (no heat shock) mRNA levels within an acclimation group (* $P < 0.05$).

and 6 hours and 12 hours of recovery (after 2 hours of heat shock) (Fig 3A). In contrast, Hsp70 mRNA in cold-acclimated AM never reached significant levels over those of controls, either during the heat shock or during recovery. Cold-acclimated AM showed significant differences among treatments (ANOVA; $P = 0.001$; Fig 3A), but these differences were because of treatment values being lower than control values. Cold-acclimated AM had significant decreases in Hsp70 mRNA levels following 1 hour of heat shock and 48 hours of recovery (after 2 hours of heat shock) (Fig 3A).

Hsp70 mRNA levels in ambient-acclimated HP were also significantly different (ANOVA; $P < 0.001$; Fig 4A). Testing of treatments vs control values indicated increases in Hsp70 mRNA levels following 1 hour and 2 hours of heat shock and 6 hours of recovery (after 2 hours of heat shock) (Fig 4A). No such induction was observed for cold-acclimated HP (ANOVA; $P = 0.082$; Fig 4A).

Hsp90 gene expression

Differences in Hsp90 gene expression were observed between acclimation groups for both AM (Fig 1) and HP (Fig 2). Similar to Hsp70 expression, Hsp90 mRNA levels in ambient-acclimated AM were significantly different (ANOVA; $P = 0.003$; Fig 3B). Animals that received 2 hours of heat shock, as well as those that received a full 2 hours of heat shock and 2 hours, 6 hours, 12 hours, or 24 hours of recovery had significant values over controls (Fig 3B). In cold-acclimated AM, Hsp90 mRNA levels were significantly different between treatments but not

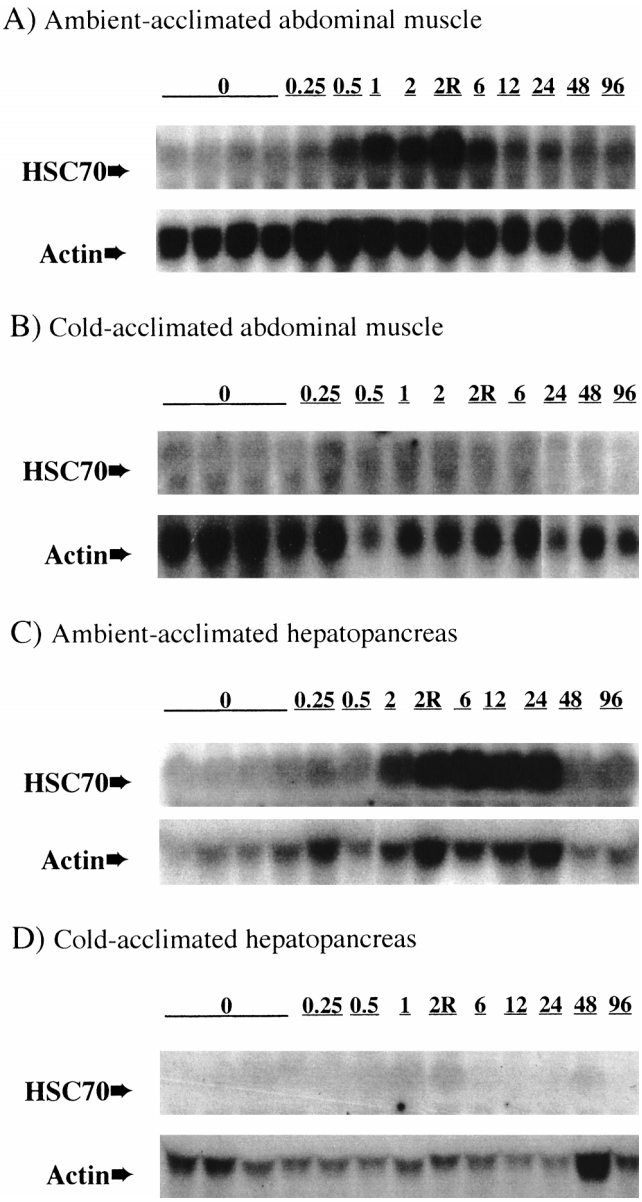

Fig 6. Northern analysis of Hsc70 expression in (A) ambient- and (B) cold-acclimated abdominal muscle, and (C) ambient- and (D) cold-acclimated hepatopancreas during heat shock and recovery. Heat shock times are indicated in hours (0.25, 0.5, 1, and 2). A 2-hour heat shock followed by a 2-hour recovery is indicated by 2R. Subsequent times (6, 12, 24, 48, 96) are hours of recovery following the 2-hour heat shock. Any missing time points were excluded because of degradation of RNA.

between treatment and control levels (ANOVA; $P = 0.017$; Fig 3B).

HP Hsp90 gene expression patterns were strikingly different between acclimation groups. Ambient-acclimated animals displayed a significant increase in HP Hsp90 mRNA over control levels at 6 hours recovery, but also a significant decrease after 48 hours recovery (ANOVA; $P < 0.001$; Fig 4B). Cold-acclimated animals showed a significant inhibition or decrease in expression from the bas-

al levels that appear to be higher than those found in ambient-acclimated controls (compare Hsp90 control mRNA levels for ambient- and cold-acclimated HP; Fig 2) (absolute quantitative assessment of these differences, however, is not available without an internal standard). Measurements of Hsp90 mRNA levels from cold-acclimated HP were significantly less than control values at the 6-hour, 12-hour, 24-hour, and 48-hour time points (ANOVA; $P < 0.001$; Fig 4B).

Polyubiquitin gene expression

For all tissues examined, under all acclimation regimes, ambient-acclimated HP was the only tissue that showed significant expression of the polyubiquitin gene (ANOVA; $P < 0.001$; Figs 4C and 5). Expression in ambient-acclimated HP was significantly different from control values at 2 hours and 6 hours recovery (after 2 hours heat shock) (Fig 4C). There were no significant changes in polyubiquitin expression in ambient-acclimated AM (ANOVA; $P = 0.90$; Fig 5). Cold-acclimated AM polyubiquitin expression was low, and we were unable to accurately quantify it (Fig 1B). Comparison of tissue-specific expression responses for ambient-acclimated AM and HP indicates that AM is a more stable tissue in response to the thermal stress we tested.

Hsc70 gene expression

The pattern of Hsc70 gene expression in ambient- vs cold-acclimated animals was consistent with those observed for the other chaperones, Hsp70 and Hsp90. Inductions of Hsc70 mRNA levels were observed in both the AM and HP of ambient-acclimated animals, and not so in the same tissues from cold-acclimated individuals, which received a heat shock over an equivalent interval (Fig 6).

Control experiments

We found no significant differences in basal molecular chaperone or polyubiquitin mRNA levels between the lobsters that acclimated in the cold room in 2-L jars (water changes once a week) and those housed in 40-L aquarium tanks (water changes every other day to simulate a flow-through system). For HP, there were no differences in Hsc70 mRNA levels, $P = 0.223$; Hsp90 mRNA levels, $P = 0.327$; or polyubiquitin mRNA levels, $P = 0.097$ (Student's *t*-test). For AM, there were no significant differences in Hsc70 mRNA levels ($P = 0.458$) or Hsp90 mRNA levels ($P = 0.486$) between the control groups (Student's *t*-test). Polyubiquitin mRNA levels were too low to quantify in AM from either control group.

Additionally, we found no significant differences in molecular chaperone or polyubiquitin mRNA levels between cold-acclimated animals that were heat shocked in the flow-through system and those that were heat shocked in the 2-L jars (HP: Hsc70 mRNA levels, $P = 0.313$; Hsp90 mRNA levels, $P = 0.370$; polyubiquitin mRNA levels, $P = 0.908$; AM: Hsc70 mRNA levels, $P = 0.973$; Hsp90 mRNA levels, $P = 0.405$) (Student's *t*-test). The lobster Hsp70 probe was the last to be hybridized with the control blots and did not bind well.

DISCUSSION

Gene expression patterns

We used homologous molecular probes to quantify *H. americanus* gene expression in vivo in different tissues and over long-term recovery periods. To our knowledge, the in vivo expression patterns of multiple stress-responsive genes from separate acclimation states have yet to be examined in the detail described here for any invertebrate, including model organisms such as *Drosophila*.

Our results illustrate how ectothermic organisms, which naturally undergo long-term seasonal acclimatization to cold temperatures, can respond uniquely to equivalent thermal shifts. The lack of molecular chaperone and polyubiquitin gene expression observed in cold-acclimated lobsters contrasts strongly with results from ambient-acclimated animals heat shocked with an equivalent temperature shift. Ambient-acclimated tissues showed significant increases in Hsp70, Hsp90, and polyubiquitin (HP) mRNA levels relative to control expression levels. In addition, similar induction patterns were evident for the cognate Hsp Hsc70. No significant increases in mRNA levels for any of these genes were observed in tissues from cold-acclimated animals relative to controls.

Tissue specific stress responses

Not all tissues respond in concert with a given thermal stress. Although both AM and HP of ambient-acclimated lobsters displayed signs of reversible protein damage (molecular chaperone expression) in response to an acute thermal stress, only the HP showed significant expression of the polyubiquitin gene, a sign of increased protein degradation or irreversible damage (Mykles 1998). This implies that AM may have been more stable than HP over the thermal interval tested. The protein pools that make up these tissues are, thus, likely to differ in their stability characteristics. Feder and Krebs (1998) reported that the gut of *Drosophila* larvae stained intensely with trypan blue dye following a severe heat shock, suggesting that it is especially thermosensitive. Interestingly, this tissue also exhibited a prolonged delay in the expression of the Hsp70 protein relative to other tissues examined. They

noted that severe heat shock markedly increased trypan blue staining in many tissues, but in inverse relationship to the number of times in which these tissues expressed Hsp70, an indication of the protective effects of this chaperone.

The consistent inhibition of Hsp90 gene expression in the HP of cold-acclimated animals during recovery from acute thermal stress may be caused by the interruption of transcriptional processing. Unlike *Hsp70,* which is known to lack introns, *Hsp90* has several introns and requires splicing to generate a complete message (Yost and Lindquist 1986). Although markedly different from the response of ambient-acclimated animals, this inhibition does indicate that the cold-acclimated animals experienced a stress in response to the shift in temperature. Observing that actin mRNA levels did not change significantly, this may relate to the roles of Hsp90 in multiple signal transduction pathways that may have reacted to the experimental treatment (Pratt 1997). For example, Hsp90 transcription in *H. americanus* is influenced by the molt cycle and in response to ecdysteroids (steroid-molting hormones) (Chang et al 1999; Spees, personal communication). The *Drosophila* ecdysteroid receptor is partly activated by Hsp90 (Arbeitman and Hogness 2000), as are most steroid receptors studied to date (Pratt 1997).

Conclusions and further questions

The observed differences in molecular chaperone and polyubiquitin gene expression between ambient- and cold-acclimated lobsters, and between tissues of ambient-acclimated animals may be the result of differences in substrates. Although the Hsp or polyubiquitin systems could themselves differ between acclimation regimes, or between tissues, it is likely that substrate flexibility, conformation, presence or absence, or concentration differences between acclimation groups or tissue types is also important. Denaturation of proteins (substrates) should precede Hsp induction or activation (or both) of ubiquitin- or proteasome-mediated degradation.

The results of our study (and those mentioned earlier) inspire several interesting questions for biochemists and ecologists alike. For a given organism, what are the first proteins to unfold? Is the identity and order in which particular proteins denature conserved between tissues, acclimatization or acclimation states, or organisms? Do multiple organisms in a given ecological community display similar protein denaturation profiles during a perturbation? Given the current state of scientific and technological progress, we are confident that future investigations in physiological ecology can address many of these exciting questions.

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